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(54) Title: CAROTENOID BIOSYNTHESIS ENZYMES

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a carotenoid biosynthetic enzyme. The invention also relates to the construction of a chimeric gene encoding all or a portion of the carotenoid biosynthetic enzyme, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the carotenoid biosynthetic enzyme in a transformed host cell.

```
SEQ ID NO:27 DFDIVLPGH
SEQ ID NO:28 MAIILVRAASPL--SAADSISHQ--GTLCQSTLLKTRFPAARWPCBLLGLAPWLAGP
SEQ ID NO:02 EEEEEENVLWGLLDAYDRCGVCAYANTFTLTQKATFERKAVW--AIYVW-CRAT
SEQ ID NO:14 HSGVLLWVSC-----GPKENINSL-VSTSCSSSGGGR--TKRFTGCSFAS-----
1 60

SEQ ID NO:27 SPAYVBSLFPVHPAGEAVVSEKRVYDVVLKQA--ALLKRLATFVLD--ARPDHMP--
SEQ ID NO:28 DELVDGPNASTYITPTALDRWEKRLDLFEGRPYDNYDAALSDTVSKFPVDIQPKDMVVG
SEQ ID NO:02 GTSATSS--AVAATETSRSSEKRVYDVVLKQA--ALVKEHKGKTKIALDLKRDVEADTH--
SEQ ID NO:14 61 120

* * * * *
SEQ ID NO:27 --LGLLEAYDRCGVCAYANTFTYL--GTMLTPDORRAIWAIVWCRATDELVDGPHAS
SEQ ID NO:28 --RNLG--KZAYDRCGVCAYANTFTYL--GTMLTPDORRAIWAIVWCRATDELVDGPHAS
SEQ ID NO:02 NMLDLNKSRIWTFDEL--YLYCYVAGTQMTFERKAVWAIYVWCRATDELVDGPHAS
SEQ ID NO:14 --NVDLLNAAAYDRCGVCAYANTFTYL--GTQMTAERKAIWAIVWCRATDELVDGPHAS
121 180

* * * * *
SEQ ID NO:27 HITPQALDRWEARLEDTFNGRFFDMLDAALSDTVSKFPVDIQPKDMVVGKMDLWKSRY
SEQ ID NO:28 YITPTALDRWEKRLDLFEGRPYDNYDAALSDTVSKFPVDIQPKDMVVGKMDLWKSRY
SEQ ID NO:02 YITPTALDRWEKRLDLFEGRPYDNYDAALSDTVSKFPVDIQPKDMVVGKMDLWKSRY
SEQ ID NO:14 HITPQALDRWEKRLSDVFEGRPYDNYDAALSHTVSKFPVDIQPKDMVVGKMDLWKSRY
181 240

* * * * *
SEQ ID NO:27 NMFDELYLYCYVAGTVGLMSVPVINGIAPESKATTESVYNAALALGIANGLTWILRDVGE
SEQ ID NO:28 NMFDELYMYCYVAGTVGLMSVPVINGIAPESKATTESVYNAALALGIANGLTWILRDVGE
SEQ ID NO:02 NMFDELYLYCYVAGTVGLMSVPVINGIAPESKATTESVYNAALALGIANGLTWILRDVGE
SEQ ID NO:14 NMFDELYLYCYVAGTVGLMSVPVINGIAPESKATTESVYNAALALGIANGLTWILRDVGE
241 300

* * * * *
SEQ ID NO:27 DARGRVYLPQDELAQAQLSDEDTAGKVTGNRIWNGKQIQARAKFFDEAKGVTELSS
SEQ ID NO:28 DARGRVYLPQDELAQAQLSDEDTAGKVTGNRIWNGKQIQARAKFFDEAKGVTELSSQ
SEQ ID NO:02 DARGRVYLPQDELAQAQLSDEDTAGKVTGNRIWNGKQIQARAKFFDEAKGVTELSSQ
SEQ ID NO:14 DARGRVYLPQDELAQAQLSDDDTAGKVTGNRIWNGKQIQARAKFFDEAKGVTELSS
301 360

* * * * *
SEQ ID NO:27 ASRWVPLASILLYRQILDSIEANDYNNFTKRAYVSKPKLLTLPYAYARAVLPPEKSTSCP
SEQ ID NO:28 ASRWVPLASILLYRQILDSIEANDYNNFTKRAYVSKPKLLTLPYAYARAVLPPEKSTSCP
SEQ ID NO:02 ASRWVPLASILLYRQILDSIEANDYNNFTKRAYVSKPKLLTLPYAYARAVLPPEKSTSCP
SEQ ID NO:14 ASRWVPLASILLYRQILDSIEANDYNNFTKRAYVSKPKLLTLPYAYARAVLPPEKSTSCP
361 420

SEQ ID NO:27 L--AKT
SEQ ID NO:28 LRNGQT
SEQ ID NO:02 -----
SEQ ID NO:14 VR--E.
421 426
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TITLE

CAROTENOID BIOSYNTHESIS ENZYMES

This application claims the benefit of U.S. Provisional Application No. 60/083,042, filed April 24, 1998.

5

FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding enzymes of the carotenoid biosynthesis pathway in plants and seeds.

BACKGROUND OF THE INVENTION

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Plant carotenoids are orange and red lipid-soluble pigments found embedded in the membranes of chloroplasts and chromoplasts. In leaves and immature fruits the color is masked by chlorophyll but in later stages of development these pigments contribute to the bright color of flowers and fruits. Carotenoids protect against photooxidation processes and harvest light for photosynthesis. The carotenoid biosynthesis pathway leads to the production of abscisic acid with intermediaries useful in the agricultural and food industries as well as products thought to be involved in cancer prevention. (Bartley, G. E., and Scolnik, P. A. (1995) *Plant Cell* 7:1027-1038).

15

Phytoene synthase carries out the first step in the carotenoid biosynthetic pathway converting geranylgeranyl diphosphate to phytoene. There are two different phytoene synthases in tomato with different expression patterns: one is expressed at higher levels in mature fruits while the other one is expressed at higher levels in leaves (Bartley, G. E., Scolnik, P.A. (1993) *J. Biol Chem.* 268:25718-25721). It has been speculated that in corn at least two different alleles of phytoene synthase should be present but only one has been identified to date (Buckner, B. et al. (1996) *Genetics* 143:479-488).

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In the next step of the carotenoid biosynthesis pathway, phytoene desaturase transforms phytoene into phytofluene. After another desaturation step, the enzyme zeta-carotene desaturase (carotene 7, 8 desaturase; EC 1.134.99.30) converts the lightly colored zeta-carotene to neurosporene which is further desaturated into lycopene. Lycopene may have one of two different fates: through the action of lycopene epsilon cyclase it may become alpha carotene, or it may be transformed into beta carotene by lycopene cyclase. Beta-carotene dehydroxylase converts beta-carotene into zeaxanthin. Zeaxanthin epoxidase transforms zeaxanthin into violxanthin and eventually abscisic acid. The genes encoding this chloroplast-imported protein have been identified in *N. plumbaginifolia*, pepper and tomato. Zeaxanthin epoxidase appears to also be involved in protection from environmental stress (Corinne A. et al. (1998) *Plant Phys.* 118:1021-1028) and uses FAD as a cofactor (Buch, K. et al. (1995) *FEBS Lett.* 376:45-48).

30

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Zeaxanthin is the bright orange product highly prized as a pigmenting agent for animal feed which makes the meat fat, skin, and egg yolks a dark yellow (Scott, M. L. et al. (1968) *Poultry Sci.* 47:863-872). Gram per gram, zeaxanthin is one of the best pigmenting

compounds because it is highly absorbable. Yellow corn, which produces one of the best ratios of lutein to zeaxanthin contains in average 20 to 25 mg of xanthophyll per kg while marigold petals yield 6,000 to 10,000 mg/kg.

SUMMARY OF THE INVENTION

5 The instant invention relates to isolated nucleic acid fragments encoding carotenoid biosynthetic enzymes. Specifically, this invention concerns an isolated nucleic acid fragment encoding a phytoene synthase or a zeaxanthin epoxidase. In addition, this invention relates to a nucleic acid fragment that is complementary to the nucleic acid fragment encoding phytoene synthase or zeaxanthin epoxidase.

10 An additional embodiment of the instant invention pertains to a polypeptide encoding all or a substantial portion of a carotenoid biosynthetic enzyme selected from the group consisting of phytoene synthase and zeaxanthin epoxidase.

15 In another embodiment, the instant invention relates to a chimeric gene encoding a phytoene synthase or a zeaxanthin epoxidase, or to a chimeric gene that comprises a nucleic acid fragment that is complementary to a nucleic acid fragment encoding a phytoene synthase or a zeaxanthin epoxidase, operably linked to suitable regulatory sequences, wherein expression of the chimeric gene results in production of levels of the encoded protein in a transformed host cell that is altered (i.e., increased or decreased) from the level produced in an untransformed host cell.

20 In a further embodiment, the instant invention concerns a transformed host cell comprising in its genome a chimeric gene encoding a phytoene synthase or a zeaxanthin epoxidase, operably linked to suitable regulatory sequences. Expression of the chimeric gene results in production of altered levels of the encoded protein in the transformed host cell. The transformed host cell can be of eukaryotic or prokaryotic origin, and include cells
25 derived from higher plants and microorganisms. The invention also includes transformed plants that arise from transformed host cells of higher plants, and seeds derived from such transformed plants.

30 An additional embodiment of the instant invention concerns a method of altering the level of expression of a phytoene synthase or a zeaxanthin epoxidase in a transformed host cell comprising: a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a phytoene synthase or a zeaxanthin epoxidase; and b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of altered levels of phytoene synthase or zeaxanthin epoxidase in the transformed host cell.

35 An addition embodiment of the instant invention concerns a method for obtaining a nucleic acid fragment encoding all or a substantial portion of an amino acid sequence encoding a phytoene synthase or a zeaxanthin epoxidase.

A further embodiment of the instant invention is a method for evaluating at least one compound for its ability to inhibit the activity of a phytoene synthase or a zeaxanthin

epoxidase, the method comprising the steps of: (a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a phytoene synthase or a zeaxanthin epoxidase, operably linked to suitable regulatory sequences; (b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein
5 expression of the chimeric gene results in production of phytoene synthase or zeaxanthin epoxidase in the transformed host cell; (c) optionally purifying the phytoene synthase or the zeaxanthin epoxidase expressed by the transformed host cell; (d) treating the phytoene synthase or the zeaxanthin epoxidase with a compound to be tested; and (e) comparing the activity of the phytoene synthase or the zeaxanthin epoxidase that has been treated with a
10 test compound to the activity of an untreated phytoene synthase or zeaxanthin epoxidase, thereby selecting compounds with potential for inhibitory activity.

BRIEF DESCRIPTION OF THE DRAWING AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description
15 and the accompanying drawing and Sequence Listing which form a part of this application.

Figure 1 depicts the amino acid sequence alignment between the phytoene synthase from corn contig assembled of clones csi1.pk0034.d8 and p0008.cb3ld95rb (SEQ ID NO:2), soybean clone sl2.pk0045.b10 (SEQ ID NO:14), *Lycopersicon esculentum* (NCBI gi
Accession No. 585747, SEQ ID NO:27) and *Zea mays* (NCBI gi Accession No. 1346883,
20 SEQ ID NO:28). Amino acids which are conserved among all sequences are indicated with an asterisk (*). Dashes are used by the program to maximize alignment of the sequences.

The following sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

25 SEQ ID NO:1 is the nucleotide sequence comprising the contig assembled from the entire cDNA insert in clone csi1.pk0034.d8 and a portion of the cDNA insert in clone p0008.cb3ld95rb encoding an entire corn phytoene synthase 2.

SEQ ID NO:2 is the deduced amino acid sequence of an entire corn phytoene synthase 2 derived from the nucleotide sequence of SEQ ID NO:1.

30 SEQ ID NO:3 is the nucleotide sequence comprising the contig assembled from a portion of the cDNA insert in clones p0121.cfrmo87r, p0091.cmarc67r and p0005.cbmej22r encoding almost half a corn phytoene synthase.

SEQ ID NO:4 is the deduced amino acid sequence of almost half a corn phytoene synthase derived from the nucleotide sequence of SEQ ID NO:3.

35 SEQ ID NO:5 is the nucleotide sequence comprising the contig assembled from a portion of the cDNA insert in clones rds1c.pk005.l5, rlr6.pk0028.g3 and rds2c.pk007.f16 encoding the N-terminal 40% of a rice phytoene synthase.

SEQ ID NO:6 is the deduced amino acid sequence of the N-terminal 40% of a rice phytoene synthase derived from the nucleotide sequence of SEQ ID NO:5.

SEQ ID NO:7 is the nucleotide sequence comprising the contig assembled from a portion of the cDNA insert in clones rl0n.pk109.j7 and rl0n.pk120.p4 encoding a portion of a rice phytoene synthase 2.

5 SEQ ID NO:8 is the deduced amino acid sequence of a portion of a rice phytoene synthase 2 derived from the nucleotide sequence of SEQ ID NO:7.

SEQ ID NO:9 is the nucleotide sequence comprising the contig assembled from the entire cDNA insert in clone rl0.pk0005.e5 and a portion of the cDNA insert in clones rca1n.pk001.18 and rlm1n.pk001.a4 encoding the C-terminal two thirds of a rice phytoene synthase.

10 SEQ ID NO:10 is the deduced amino acid sequence of the C-terminal two thirds of a rice phytoene synthase derived from the nucleotide sequence of SEQ ID NO:9.

SEQ ID NO:11 is the nucleotide sequence comprising the entire cDNA insert in clone sl1.pk0029.h5 encoding the C-terminal two thirds of a soybean phytoene synthase 2.

15 SEQ ID NO:12 is the deduced amino acid sequence of the C-terminal two thirds of a soybean phytoene synthase 2 derived from the nucleotide sequence of SEQ ID NO:11.

SEQ ID NO:13 is the nucleotide sequence comprising the entire cDNA insert in clone sl2.pk0045.b10 encoding an entire soybean phytoene synthase.

SEQ ID NO:14 is the deduced amino acid sequence of an entire soybean phytoene synthase derived from the nucleotide sequence of SEQ ID NO:13.

20 SEQ ID NO:15 is the nucleotide sequence comprising the entire cDNA insert in clone wr1.pk0139.g3 encoding the C-terminal two thirds of a wheat phytoene synthase 2.

SEQ ID NO:16 is the deduced amino acid sequence of the C-terminal two thirds of a wheat phytoene synthase 2 derived from the nucleotide sequence of SEQ ID NO:15.

25 SEQ ID NO:17 is the nucleotide sequence comprising the contig assembled from the entire cDNA insert in clone cbn2.pk0051.e8 and a portion of the cDNA insert in clones p0031.ccmaj44r and p0097.cqrag63r encoding a portion of a corn zeaxanthin epoxidase.

SEQ ID NO:18 is the deduced amino acid sequence of a portion of a corn zeaxanthin epoxidase derived from the nucleotide sequence of SEQ ID NO:17.

30 SEQ ID NO:19 is the nucleotide sequence comprising the contig assembled from the entire cDNA insert in clone cr1n.pk0033.d8 and a portion of the cDNA insert in clones p0110.cgsmpp01r, p0012.cglae05r and p0088.clrim55r encoding the C-terminal half of a corn zeaxanthin epoxidase.

SEQ ID NO:20 is the deduced amino acid sequence of the C-terminal half of a corn zeaxanthin epoxidase derived from the nucleotide sequence of SEQ ID NO:19.

35 SEQ ID NO:21 is the nucleotide sequence comprising the entire cDNA insert in clone sl1.pk0015.c4 encoding a portion of a soybean zeaxanthin epoxidase.

SEQ ID NO:22 is the deduced amino acid sequence of a portion of a soybean zeaxanthin epoxidase derived from the nucleotide sequence of SEQ ID NO:21.

SEQ ID NO:23 is the nucleotide sequence comprising the 5'-terminal portion of the cDNA insert in clone sl2.pk0109.b6 encoding the N-terminal three quarters of a soybean zeaxanthin epoxidase.

5 SEQ ID NO:24 is the deduced amino acid sequence of the N-terminal three quarters of a soybean zeaxanthin epoxidase. derived from the nucleotide sequence of SEQ ID NO:23.

SEQ ID NO:25 is the nucleotide sequence comprising the 3'-terminal portion of the cDNA insert in clone sl2.pk0109.b6 encoding the C-terminal fifth of a soybean zeaxanthin epoxidase.

10 SEQ ID NO:26 is the deduced amino acid sequence of the C-terminal fifth of a soybean zeaxanthin epoxidase derived from the nucleotide sequence of SEQ ID NO:25.

SEQ ID NO:27 is the amino acid sequence of a *Lycopersicon esculentum* phytoene synthase, NCBI gi Accession No. 585747.

SEQ ID NO:28 is the amino acid sequence of a *Cucumis melo* phytoene synthase, NCBI gi Accession No. 1346882.

15 The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Research* 13:3021-3030 (1985) and in the *Biochemical Journal* 219 (No. 2):345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

20 DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An
25 isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA. As used herein, "contig" refers to an assemblage of overlapping nucleic acid sequences to form one contiguous nucleotide sequence. For example, several DNA sequences can be compared and aligned to identify common or overlapping regions. The individual sequences can then be assembled into a
30 single contiguous nucleotide sequence.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more
35 nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate alteration of gene

expression by antisense or co-suppression technology or alteration of the functional properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less than the entire coding region of a gene, and by nucleic acid fragments that do not share 100% sequence identity with the gene to be suppressed. Moreover, alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded protein, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the nucleic acid fragments disclosed herein.

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent similarity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Preferred are those nucleic acid fragments whose nucleotide sequences encode amino acid sequences that are 80% similar to the amino acid sequences reported herein. More preferred nucleic acid fragments encode amino acid sequences that are 90% similar to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are 95% similar to the amino acid sequences reported herein. Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the amino acid sequences was performed using the Clustal method of alignment (Higgins, D. G. and Sharp, P. M. (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the

sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol.* 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches partial or complete amino acid and nucleotide sequences encoding one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the phytoene synthase or the zeaxanthin epoxidase proteins as set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled

artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

“Gene” refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. “Native gene” refers to a gene as found in nature with its own regulatory sequences. “Chimeric gene” refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. “Endogenous gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

“Coding sequence” refers to a DNA sequence that codes for a specific amino acid sequence. “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

“Promoter” refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg, (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a DNA sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D. (1995) *Molecular Biotechnology* 3:225).

The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) *Plant Cell* 1:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Pat. No. 5,107,065, incorporated herein by reference). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or

non-transformed organisms. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference).

5 "Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular
10 localization signals.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an
15 amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels, J. J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal
20 peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys.* 100:1627-1632).

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of
25 methods of plant transformation include Agrobacterium-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or "gene gun" transformation technology (Klein T. M. et al. (1987) *Nature (London)* 327:70-73; U.S. Patent No. 4,945,050).

Standard recombinant DNA and molecular cloning techniques used herein are well
30 known in the art and are described more fully in Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Maniatis").

Nucleic acid fragments encoding at least a portion of several carotenoid biosynthetic enzymes have been isolated and identified by comparison of random plant cDNA sequences
35 to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. Table 1 lists the proteins that are described herein, and the designation of the cDNA clones that comprise the nucleic acid fragments encoding these proteins.

TABLE 1
Carotenoid Biosynthetic Enzymes

| Enzyme | Clone | Plant |
|----------------------|--|---------|
| Phytoene Synthase | Contig of: p0008.cb3ld95rb csi1.pk0034.d8 | Corn |
| | Contig of: p0121.cfrmo87r p0091.cmarc67r p0005.cbmej22r | Corn |
| | Contig of: rds1c.pk005.l5 rlr6.pk0028.g3 rds2c.pk007.fl6 | Rice |
| | Contig of: rl0n.pk109.j7 rl0n.pk120.p4 | Rice |
| | Contig of: rlm1n.pk001.a4 rcal1n.pk001.l8 rl0.pk0005.e5 | Rice |
| | sl1.pk0029.h5 | Soybean |
| | sl2.pk0045.b10 | Soybean |
| | wrl.pk0139.g3 | Wheat |
| | contig of: cbn2.pk0051.e8 p0031.ccmaj44r p0097.cqrag63r | Corn |
| | Contig of: p0110.cgsmmp01r p0012.cglae05r p0088.clrim55r crl1n.pk0033.d8 | Corn |
| Zeaxanthin Epoxidase | sl1.pk0015.c4 | Soybean |
| | sl2.pk0109.b6 | Soybean |

The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding other phytoene synthases or zeaxanthin epoxidases, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the

instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., (1988) *Proc. Natl. Acad. Sci. USA* 85:8998) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., (1989) *Proc. Natl. Acad. Sci. USA* 86:5673; Loh et al., (1989) *Science* 243:217). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman, M. A. and Martin, G. R., (1989) *Techniques* 1:165).

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner, R. A. (1984) *Adv. Immunol.* 36:1; Maniatis).

The nucleic acid fragments of the instant invention may be used to create transgenic plants in which the disclosed phytoene synthase or zeaxanthin epoxidase are present at higher or lower levels than normal or in cell types or developmental stages in which they are not normally found. This would have the effect of altering the level of lycopene or zeaxanthin in those cells. Because the nucleotide sequence of corn clone cs1l.pk0034.d8 is

so divergent from known phytoene synthase genes it may be possible to overexpress it in transgenic plants without causing co-suppression. Co-suppression of phytoene synthase in rice may re-direct the carbon flux towards tocopherol biosynthesis to improve the grain eating qualities. Manipulation of the levels of zeaxanthin epoxidase in transgenic corn may result in higher levels of zeaxanthin, an important ingredient in animal feed.

Overexpression of the phytoene synthase or the zeaxanthin epoxidase proteins of the instant invention may be accomplished by first constructing a chimeric gene in which the coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of development. For reasons of convenience, the chimeric gene may comprise promoter sequences and translation leader sequences derived from the same genes. 3' Non-coding sequences encoding transcription termination signals may also be provided. The instant chimeric gene may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant chimeric gene can then be constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J.* 4:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant carotenoid biosynthetic enzyme to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that the chimeric gene described above may be further supplemented by altering the coding sequence to encode phytoene synthase or zeaxanthin epoxidase with appropriate intracellular targeting sequences such as transit sequences (Keegstra, K. (1989) *Cell* 56:247-253), signal sequences or sequences encoding endoplasmic reticulum localization (Chrispeels, J. J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53), or nuclear localization signals (Raikhel, N. (1992) *Plant Phys.* 100:1627-1632) added and/or with targeting sequences that are already present removed. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of utility may be discovered in the future.

It may also be desirable to reduce or eliminate expression of genes encoding phytoene synthase or zeaxanthin epoxidase in plants for some applications. In order to accomplish this, a chimeric gene designed for co-suppression of the instant carotenoid biosynthetic enzyme can be constructed by linking a gene or gene fragment encoding a phytoene

synthase or a zeaxanthin epoxidase to plant promoter sequences. Alternatively, a chimeric gene designed to express antisense RNA for all or part of the instant nucleic acid fragment can be constructed by linking the gene or gene fragment in reverse orientation to plant promoter sequences. Either the co-suppression or antisense chimeric genes could be introduced into plants via transformation wherein expression of the corresponding endogenous genes are reduced or eliminated.

The instant phytoene synthase or zeaxanthin epoxidase (or portions thereof) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to these proteins by methods well known to those skilled in the art. The antibodies are useful for detecting phytoene synthase or zeaxanthin epoxidase *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant phytoene synthase or zeaxanthin epoxidase are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a chimeric gene for production of the instant phytoene synthase or zeaxanthin epoxidase. This chimeric gene could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded carotenoid biosynthetic enzyme. An example of a vector for high level expression of the instant phytoene synthase or zeaxanthin epoxidase in a bacterial host is provided (Example 7).

Additionally, the instant phytoene synthase or zeaxanthin epoxidase can be used as targets to facilitate design and/or identification of inhibitors of those enzymes that may be useful as herbicides. This is desirable because the phytoene synthase or the zeaxanthin epoxidase described herein catalyze various steps in carotenoid biosynthesis. Accordingly, inhibition of the activity of one or more of the enzymes described herein could lead to inhibition plant growth. Thus, the instant phytoene synthase or zeaxanthin epoxidase could be appropriate for new herbicide discovery and design.

All or a substantial portion of the nucleic acid fragments of the instant invention may also be used as probes for genetically and physically mapping the genes that they are a part of, and as markers for traits linked to those genes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes. For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with the nucleic acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et al., (1987) *Genomics* 1:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted

and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein, D. et al., (1980) *Am. J. Hum. Genet.* 32:314-331).

5 The production and use of plant gene-derived probes for use in genetic mapping is described in R. Bernatzky, R. and Tanksley, S. D. (1986) *Plant Mol. Biol. Reporter* 4(1):37-41. Numerous publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2 intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to
10 those skilled in the art.

Nucleic acid probes derived from the instant nucleic acid sequences may also be used for physical mapping (i.e., placement of sequences on physical maps; see Hoheisel, J. D., et al., In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

15 In another embodiment, nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping (Trask, B. J. (1991) *Trends Genet.* 7:149-154). Although current methods of FISH mapping favor use of large clones (several to several hundred KB; see Laan, M. et al. (1995) *Genome Research* 5:13-20), improvements in sensitivity may allow performance of FISH mapping
20 using shorter probes.

A variety of nucleic acid amplification-based methods of genetic and physical mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification (Kazazian, H. H. (1989) *J. Lab. Clin. Med.* 114(2):95-96), polymorphism of PCR-amplified fragments (CAPS; Sheffield, V. C. et al. (1993) *Genomics* 25 16:325-332), allele-specific ligation (Landegren, U. et al. (1988) *Science* 241:1077-1080), nucleotide extension reactions (Sokolov, B. P. (1990) *Nucleic Acid Res.* 18:3671), Radiation Hybrid Mapping (Walter, M. A. et al. (1997) *Nature Genetics* 7:22-28) and Happy Mapping (Dear, P. H. and Cook, P. R. (1989) *Nucleic Acid Res.* 17:6795-6807). For these methods, the sequence of a nucleic acid fragment is used to design and produce primer pairs for use in
30 the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the instant nucleic acid sequence. This, however, is generally not necessary for mapping methods.

35 Loss of function mutant phenotypes may be identified for the instant cDNA clones either by targeted gene disruption protocols or by identifying specific mutants for these genes contained in a maize population carrying mutations in all possible genes (Ballinger and Benzer, (1989) *Proc. Natl. Acad. Sci USA* 86:9402; Koes et al., (1995) *Proc. Natl. Acad. Sci USA* 92:8149; Bensen et al., (1995) *Plant Cell* 7:75). The latter approach may be

accomplished in two ways. First, short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols in conjunction with a mutation tag sequence primer on DNAs prepared from a population of plants in which Mutator transposons or some other mutation-causing DNA element has been introduced (see Bensen, *supra*). The amplification of a specific DNA fragment with these primers indicates the insertion of the mutation tag element in or near the plant gene encoding the phytoene synthase or the zeaxanthin epoxidase. Alternatively, the instant nucleic acid fragment may be used as a hybridization probe against PCR amplification products generated from the mutation population using the mutation tag sequence primer in conjunction with an arbitrary genomic site primer, such as that for a restriction enzyme site-anchored synthetic adaptor. With either method, a plant containing a mutation in the endogenous gene encoding a phytoene synthase or a zeaxanthin epoxidase can be identified and obtained. This mutant plant can then be used to determine or confirm the natural function of the phytoene synthase or the zeaxanthin epoxidase gene product.

EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various corn, rice, soybean and wheat tissues were prepared. The characteristics of the libraries are described below.

TABLE 2

cDNA Libraries from Corn, Rice, Soybean and Wheat

| Library | Tissue | Clone |
|---------|---|-----------------|
| cbn2 | Corn Developing Kernel Two Days After Pollination | cbn2.pk0051.e8 |
| cr1n | Corn Root From 7 Day Old Seedlings* | cr1n.pk0033.d8 |
| csi1 | Corn Silk | csi1.pk0034.d8 |
| p0005 | Corn Immature Ear | p0005.cbmej22r |
| p0008 | Corn Leaf, 3-Weeks-Old | p0008.cb3ld95rb |
| p0012 | Corn Middle 3/4 of the 3rd Leaf Blade and Mid Rib From Green Leaves Treated with Jasmonic Acid (1 mg/ml in 0.02% Tween 20) for 24 Hours Before Collection | p0012.cglae05r |
| p0031 | Corn Shoot Culture | p0031.ccmaj44r |

| Library | Tissue | Clone |
|---------|---|-----------------|
| p0088 | Corn Leaf From Mutant Plant** Prior to Genetic Lesion Formation | p0088.clim55r |
| p0091 | Corn Roots 2 and 3 Days After Germination, Pooled | p0091.cmarc67r |
| p0097 | Corn V9 Whorl Section (7 cm) From Plant Infected Four Times With European Corn Borer | p0097.cqrag63r |
| p0110 | Corn (Stages V3/V4) Leaf Tissue Minus Midrib Harvested 4 Hours, 24 Hours and 7 Days After Infiltration With Salicylic Acid, Pooled* | p0110.cgsmp01r |
| p0121 | Corn Shank Ear Tissue Collected 5 Days After Pollination* | p0121.cfrmo87r |
| rca1n | Rice Callus* | rca1n.pk001.l8 |
| rds1c | Rice Developing Seeds | rds1c.pk005.l5 |
| rds2c | Rice Developing Seeds From Middle of the Plant | rds2c.pk007.f16 |
| rl0 | Rice 15 Day Old Leaf | rl0.pk0005.e5 |
| rl0n | Rice 15 Day Old Leaf* | rl0n.pk109.j7 |
| | | rl0n.pk120.p4 |
| rlm1n | Rice Leaf 15 Days After Germination Harvested 2-72 Hours Following Infection With <i>Magnaporthe grisea</i> (4360-R-62 and 4360-R-67) Normalized at 30 Degrees C for 24 Hours Using 10 Fold Excess Driver | rlm1n.pk001.a4 |
| rls6 | Rice Leaf 15 Days After Germination, 6 Hours After Infection of Strain <i>Magaporthe grisea</i> 4360-R-67 (AVR2-YAMO); Susceptible | rlr6.pk0028.g3 |
| sl1 | Soybean Two-Week-Old Developing Seedlings | sl1.pk0015.c4 |
| | | sl1.pk0029.h5 |
| sl2 | Soybean Two-Week-Old Developing Seedlings Treated With 2.5 ppm chlorimuron | sl2.pk0045.b10 |
| | | sl2.pk0109.b6 |
| wr1 | Wheat Root From 7 Day Old Seedling | wr1.pk0139.g3 |

*These libraries were normalized essentially as described in U.S. Patent No. 5,482,845

**Simmons, C. et al. (1998) *Mol. Plant Microbe Interact.* 11:1110-1118

- 5 cDNA libraries were prepared in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). Conversion of the Uni-ZAP™ XR libraries into plasmid libraries was accomplished according to the protocol provided by Stratagene. Upon conversion, cDNA inserts were contained in the plasmid vector pBluescript. cDNA inserts from randomly picked bacterial colonies containing
- 10 recombinant pBluescript plasmids were amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences or plasmid DNA was prepared from cultured bacterial cells. Amplified insert DNAs or plasmid DNAs were sequenced in dye-primer sequencing reactions to generate partial cDNA sequences

(expressed sequence tags or "ESTs"; see Adams, M. D. et al., (1991) *Science* 252:1651). The resulting ESTs were analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 2

Identification of cDNA Clones

5 ESTs encoding carotenoid biosynthetic enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol.* 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. (1993) *Nature Genetics* 3:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

EXAMPLE 3

Characterization of cDNA Clones Encoding Phytoene Synthase

The BLASTX search using the EST sequences from clones csl1.pk0034.d8, ssm.pk0011.d9, sl1.pk0069.e4, sl1.pk0029.h5, sl1.pk0073.g10, sl1.pk0031.b8 and wr1.pk0139.g3 revealed similarity of the proteins encoded by the cDNAs to Phytoene Synthase from corn, *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Cucumis melo*, and *Capsicum annum* (GenBank Accession Nos. U32636, L25812, L23424, Z37543, X68017 respectively). Further analysis of the sequences from clones ssm.pk0011.d9 and sl1.pk0069.e4 revealed a significant region of overlap, thus affording the assembly of a contig encoding a portion of a soybean Phytoene Synthase. Likewise, further analysis of the sequences from clones sl1.pk0029.h5 and sl1.pk0073.g10 revealed a significant region of overlap, thus affording the assembly of an additional contig encoding a portion of a soybean Phytoene Synthase. The BLAST results for each of these ESTs and contigs are shown in Table 3:

TABLE 3
**BLAST Results for Clones Encoding Polypeptides Homologous
to Phytoene Synthase**

| Clone | Organism | GenBank Accession No. | BLAST pLog Score |
|---|--------------------------------|--------------------------|------------------|
| csi1.pk0034.d8 | <i>Maize</i> | U32636 | 33.00 |
| Contig of: ssm.pk0011.d9 sl1.pk0069.e4 | <i>Arabidopsis thaliana</i> | L25812 | 54.40 |
| Contig of: sl1.pk0029.h5 sl1.pk0073.g10 | <i>Lycopersicon esculentum</i> | L23424 | 20.00 |
| sl1.pk0031.b8 | <i>Cucumis melo</i> | Z37543 | 50.00 |
| wrl.pk0139.g3 | <i>Capsicum annum</i> | X68017 | 31.70 |

- 5 TBLASTN analysis of the proprietary plant EST database indicated that other corn rice and soybean clones besides those mentioned above encoded phytoene synthetase. The BLASTX search using the nucleotide sequences of the contig assembled from a portion of the cDNA insert in clones p0121.cfrmo87r, p0091.cmarc67r and p0005.cbmej22r revealed similarity of the proteins encoded by the cDNAs to phytoene synthase from *Capsicum*
- 10 *annuum* (NCBI gi Accession No. 585749). The BLASTX search using the nucleotide sequences of the contig assembled from a portion of the cDNA insert in clones rds1c.pk005.l5, rlr6.pk0028.g3 and rds2c.pk007.fl6 and of the contig assembled from the entire cDNA insert in clone rl0.pk0005.e5 and a portion of the cDNA insert in clones rlm1n.pk001.a4 and rca1n.pk001.l8 revealed similarity of the proteins encoded by the
- 15 cDNAs to phytoene synthase from *Zea mays* (NCBI gi Accession No. 1346883). The BLASTX search using the nucleotide sequences from the contig assembled of a portion of the cDNA insert in clones rl0n.pk109.j7 and rl0n.pk120.p4 revealed similarity of the proteins encoded by the cDNAs to phytoene synthase 2 from *Lycopersicon esculentum* (NCBI gi Accession No. 585747). BLASTX search using the nucleotide sequences from the
- 20 entire cDNA insert in clone sl2.pk0045.b10 revealed similarity of the proteins encoded by the cDNAs to phytoene synthase from *Narcissus pseudonarcissus* (NCBI gi Accession No. 1709885). The BLAST results for each of these sequences are shown in Table 4:

TABLE 4
BLAST Results for Clones Encoding Polypeptides Homologous
to Phytoene Synthase

| Clone | Organism | NCBI gi Accession No. | BLAST pLog Score |
|---|----------------------------------|--------------------------|---------------------|
| Contig of: p0121.cfrmo87r p0091.cmarc67r p0005.cbmej22r | <i>Capsicum annuum</i> | 585749 | 89.22 |
| Contig of: rds1c.pk005.l5 rlr6.pk0028.g3 rds2c.pk007.f16 | <i>Zea mays</i> | 1346883 | 54.22 |
| Contig of: rl0n.pk109.j7 rl0n.pk120.p4 | <i>Lycopersicon esculentum</i> | 585747 | 54.30 |
| Contig of: rlm1n.pk001.a4 rca1n.pk001.l8 rl0.pk0005.e5 | <i>Zea mays</i> | 1346883 | 132.0 |
| sl2.pk0045.b10 | <i>Narcissus pseudonarcissus</i> | 1709885 | 176.0 |

- 5 The sequence of the entire cDNA insert in clone cs11.pk0034.d8 was determined and a contig assembled with this sequence and a portion of the cDNA insert from clone p0008.cb31d95rb. The sequence of this contig is shown in SEQ ID NO:1; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:2. The amino acid sequence set forth in SEQ ID NO:2 was evaluated by BLASTP, yielding a pLog value of 132.0 versus the
- 10 *Lycopersicon esculentum* phytoene synthase 2 sequence (NCBI gi Accession No. 585747; SEQ ID NO:27). The sequence of the contig assembled of a portion of the cDNA insert from clones p0121.cfrmo87r, p0091.cmarc67r and p0005.cbmej22r is shown in SEQ ID NO:3; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:4. The sequence of the contig assembled of a portion of the cDNA insert from clones
- 15 rds1c.pk005.l5, rlr6.pk0028.g3 and rds2c.pk007.f16 is shown in SEQ ID NO:5; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:6. The sequence of the contig assembled of a portion of the cDNA insert from clones rl0n.pk109.j7 and rl0n.pk120.p4 is shown in SEQ ID NO:7; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:8. The sequence of the contig assembled from the entire cDNA insert in clone
- 20 rl0.pk0005.e5 and a portion of the cDNA insert from clones rlm1n.pk001.a4 and rca1n.pk001.l8 is shown in SEQ ID NO:9; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:10. The sequence of the entire cDNA insert in clone sl1.pk0029.h5 was determined and is shown in SEQ ID NO:11; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:12. The EST sequences from clones ssm.pk0011.d9,
- 25 sl1.pk0069.e4 and sl1.pk0073.g10 are included in the sequence from the entire cDNA insert

in clone sl1.pk0029.h5. The amino acid sequence set forth in SEQ ID NO:12 was evaluated by BLASTP, yielding a pLog value of 114.0 versus the *Cucumis melo* sequence (NCBI gi Accession No. 1346882). The sequence of the entire cDNA insert in clone sl2.pk0045.b10 was determined and is shown in SEQ ID NO:13; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:14. The EST sequences from clone sl1.pk0031.b8 is included in the sequence of the entire cDNA insert from clone sl2.pk0045.b10. The amino acid sequence set forth in SEQ ID NO:14 was evaluated by BLASTP, yielding a pLog value of 153.0 versus the *Cucumis melo* sequence. The sequence of the entire cDNA insert in clone wr1.pk0139.g3 was determined and is shown in SEQ ID NO:15; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:16. The amino acid sequence set forth in SEQ ID NO:16 was evaluated by BLASTP, yielding a pLog value of 118.0 versus the *Lycopersicon esculentum* sequence. Figure 1 presents an alignment of the amino acid sequences set forth in SEQ ID NOs:2 and 14 with the *Lycopersicon esculentum* sequence (SEQ ID NO:27) and the *Cucumis melo* sequence (SEQ ID NO:28). The data in Table 5 presents a calculation of the percent similarity of the amino acid sequences set forth in SEQ ID NOs:2 and 14 with the *Lycopersicon esculentum* sequence (SEQ ID NO:27) and the *Cucumis melo* sequence (SEQ ID NO:28).

TABLE 5

Percent Similarity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to Phytoene Synthase

| Clone | SEQ ID NO. | Percent Similarity to | |
|---|------------|-----------------------|--------|
| | | 1346882 | 585747 |
| Contig of: p0008.cb3ld95rb csi1.pk0034.d8 | 2 | 57.0 | 78.1 |
| Contig of: p0121.cfrmo87r p0091.cmarc67r p0005.cbmej22r | 4 | 70.4 | 74.2 |
| Contig of: rds1c.pk005.l5 rlr6.pk0028.g3 rds2c.pk007.fl6 | 6 | 47.6 | 32.3 |
| Contig of: rl0n.pk109.j7 rl0n.pk120.p4 | 8 | 82.4 | 82.4 |

| Clone | SEQ ID NO. | Percent Similarity to | |
|--|------------|-----------------------|--------|
| | | 1346882 | 585747 |
| Contig of: rlm1n.pk001.a4 rcal1n.pk001.l8 rl0.pk0005.e5 | 10 | 77.0 | 77.8 |
| sl1.pk0029.h5 | 12 | 77.1 | 78.7 |
| sl2.pk0045.b10 | 14 | 66.8 | 78.4 |
| wrl.pk0139.g3 | 16 | 78.7 | 81.1 |

Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the amino acid sequences was performed using the Clustal method of alignment (Higgins, D.G. and Sharp, P.M. (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10).

Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragments encode entire or nearly entire corn and soybean phytoene synthase and portions of corn, rice, soybean and wheat phytoene synthase isozymes. These sequences represent the first rice, soybean and wheat sequences encoding phytoene synthase, an entire corn variant which is 55.7% similar to the corn sequences available in the art (NCBI gi Accession Nos. 1346883 and 1098665) and a portion of a corn variant which is 72.0% similar to the art sequences.

EXAMPLE 4

Characterization of cDNA Clones Encoding Zeaxanthin Epoxidase

The BLASTX search using the nucleotide sequences from clones cbn2.pk0051.e8 and cr1n.pk0033.d8, and the EST sequences from clone sl1.pk0015.c4 revealed similarity of the proteins encoded by the cDNAs to Zeaxanthin Epoxidase from *Lycopersicon esculentum* and *Nicotiana glauca* (GenBank Accession Nos. Z83835 and X95732, respectively). The BLAST results for each of these sequences are shown in Table 6:

TABLE 6

BLASTn Results for Clones Encoding Polypeptides Homologous to Zeaxanthin Epoxidase

| Clone | Organism | GenBank Accession No. | BLAST pLog Score |
|----------------|--------------------------------|-----------------------|------------------|
| cbn2.pk0051.e8 | <i>Lycopersicon esculentum</i> | Z83835 | 45.52 |
| cr1n.pk0033.d8 | <i>Nicotiana glauca</i> | X95732 | 65.70 |
| sl1.pk0015.c4 | <i>Lycopersicon esculentum</i> | Z83835 | 8.30 |

TBLASTN analysis of the proprietary plant EST database indicated that another soybean clone besides sl1.pk0015.c4 also encoded zeaxanthin epoxidase. The BLASTX search using the EST sequences from the 5'terminal and 3'terminal portions of the cDNA insert in clone sl2.pk0109.b6 revealed similarity of the proteins encoded by the cDNAs to
 5 zeaxanthin epoxidase from *Prunus armeniaca* (NCBI gi Accession No. 3264757), with pLog values of >254 and 41.70, respectively.

The sequence of the entire cDNA insert in clone cbn2.pk0051.e8 was determined and a contig assembled with this sequence and a portion of the cDNA insert from clones p0031.ccmaj44r and p0097.cqrag63r. The nucleotide sequence of this contig is shown in
 10 SEQ ID NO:17; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:18. The sequence of the entire cDNA insert in clone cr1n.pk0033.d8 was determined and a contig assembled with this sequence and a portion of the cDNA insert from clones p0110.cgsmmp01r, p0012.cglae05r and p0088.clim55r. The nucleotide sequence of this contig is shown in SEQ ID NO:19; the deduced amino acid sequence of this cDNA is shown
 15 in SEQ ID NO:20. The sequence of the entire cDNA insert in clone sl1.pk0015.c4 was determined and is shown in SEQ ID NO:21; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:22. The sequence of the 5'terminus of the cDNA insert in clone sl2.pk0109.b6 was determined and is shown in SEQ ID NO:23; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:24. The sequence of the 3'terminus of the
 20 cDNA insert in clone sl2.pk0109.b6 was determined and is shown in SEQ ID NO:25; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:26.

The data in Table 7 presents a calculation of the percent similarity of the amino acid sequences set forth in SEQ ID NOs:18, 20, 22, 24 and 26 and the *Lycopersicon esculentum* and *Prunus armeniaca* sequences.
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TABLE 7

Percent Similarity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to Zeaxanthin Epoxidase

| Clone | SEQ ID NO. | Percent Identity to | |
|--|------------|---------------------|---------|
| | | 1772985 | 3264757 |
| Contig of: cbn2.pk0051.e8 p0031.ccmaj44r p0097.cqrag63r | 18 | 55.1 | 56.6 |
| Contig of: p0110.cgsmmp01r p0012.cglae05r p0088.clim55r cr1n.pk0033.d8 | 20 | 66.5 | 64.9 |
| sl1.pk0015.c4 | 22 | 51.9 | 51.9 |
| 5'end of sl2.pk0109.b6 | 24 | 66.1 | 72.7 |
| 3'end of sl2.pk0109.b6 | 26 | | |

Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the amino acid sequences was performed using the Clustal method of alignment (Higgins, D. G. and Sharp, P. M. (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10).

Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragments encode entire or nearly entire soybean zeaxanthin epoxidase and portions of corn and soybean zeaxanthin epoxidase isozymes. These sequences represent the first corn and soybean sequences encoding zeaxanthin epoxidase.

EXAMPLE 5

Expression of Chimeric Genes in Monocot Cells

A chimeric gene comprising a cDNA encoding a carotenoid biosynthetic enzyme in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (Nco I or Sma I) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes Nco I and SmaI and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb Nco I-Sma I fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from pML103 contains a 1.05 kb Sal I-Nco I promoter fragment of the maize 27 kD zein gene and a 0.96 kb Sma I-Sal I fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding a carotenoid biosynthetic enzyme, and the 10 kD zein 3' region.

The chimeric gene described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed

with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al., (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein T. M. et al., (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 µm in diameter) are coated with DNA using the following technique. Ten µg of plasmid DNAs are added to 50 µL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 µL of a 2.5 M solution) and spermidine free base (20 µL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 µL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 µL of ethanol. An aliquot (5 µL) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains gluphosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter

of actively growing callus can be identified on some of the plates containing the glufosinate-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

- Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., (1990) *Bio/Technology* 8:833-839).

EXAMPLE 6

Expression of Chimeric Genes in Dicot Cells

- A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the β subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* 261:9228-9238) can be used for expression of the instant carotenoid biosynthetic enzyme in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

- The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

- Soybean embryos may then be transformed with the expression vector comprising sequences encoding a carotenoid biosynthetic enzyme. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

- Soybean embryogenic suspension cultures can maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein T. M. et al. (1987) *Nature* (London) 327:70-73, U.S.

Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al.(1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the carotenoid biosynthetic enzyme and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL DNA (1 µg/µL), 20 µl spermidine (0.1 M), and 50 µL CaCl₂ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µL 70% ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five µL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

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EXAMPLE 7

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant carotenoid biosynthetic enzymes can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene* 56:125-135) which employs the bacteriophage T7 RNA polymerase/T7

promoter system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 µg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 µL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 µg/mL ampicillin. Transformants containing the gene encoding the carotenoid biosynthetic enzyme are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol.* 189:113-130). Cultures are grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio-β-galactoside, the inducer) can be added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by centrifugation and re-suspended in 50 µL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One µg of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

EXAMPLE 8

Evaluating Compounds for Their Ability to Inhibit the Activity of Carotenoid Biosynthetic Enzymes

The carotenoid biosynthetic enzymes described herein may be produced using any
5 number of methods known to those skilled in the art. Such methods include, but are not
limited to, expression in bacteria as described in Example 7, or expression in eukaryotic cell
culture, *in planta*, and using viral expression systems in suitably infected organisms or cell
lines. The instant carotenoid biosynthetic enzymes may be expressed either as mature forms
10 of the proteins as observed *in vivo* or as fusion proteins by covalent attachment to a variety
of enzymes, proteins or affinity tags. Common fusion protein partners include glutathione
S-transferase ("GST"), thioredoxin ("Trx"), maltose binding protein, and C- and/or
N-terminal hexahistidine polypeptide ("His₆"). The fusion proteins may be engineered
with a protease recognition site at the fusion point so that fusion partners can be separated by
15 protease digestion to yield intact mature enzyme. Examples of such proteases include
thrombin, enterokinase and factor Xa. However, any protease can be used which specifically
cleaves the peptide connecting the fusion protein and the enzyme.

Purification of the instant carotenoid biosynthetic enzymes, if desired, may utilize any
number of separation technologies familiar to those skilled in the art of protein purification.
Examples of such methods include, but are not limited to, homogenization, filtration,
20 centrifugation, heat denaturation, ammonium sulfate precipitation, desalting, pH
precipitation, ion exchange chromatography, hydrophobic interaction chromatography and
affinity chromatography, wherein the affinity ligand represents a substrate, substrate analog
or inhibitor. When the carotenoid biosynthetic enzymes are expressed as fusion proteins, the
purification protocol may include the use of an affinity resin which is specific for the fusion
25 protein tag attached to the expressed enzyme or an affinity resin containing ligands which
are specific for the enzyme. For example, a carotenoid biosynthetic enzyme may be
expressed as a fusion protein coupled to the C-terminus of thioredoxin. In addition, a (His)₆
peptide may be engineered into the N-terminus of the fused thioredoxin moiety to afford
additional opportunities for affinity purification. Other suitable affinity resins could be
30 synthesized by linking the appropriate ligands to any suitable resin such as Sepharose-4B. In
an alternate embodiment, a thioredoxin fusion protein may be eluted using dithiothreitol;
however, elution may be accomplished using other reagents which interact to displace the
thioredoxin from the resin. These reagents include β -mercaptoethanol or other reduced
thiol. The eluted fusion protein may be subjected to further purification by traditional means
35 as stated above, if desired. Proteolytic cleavage of the thioredoxin fusion protein and the
enzyme may be accomplished after the fusion protein is purified or while the protein is still
bound to the ThioBond™ affinity resin or other resin.

Crude, partially purified or purified enzyme, either alone or as a fusion protein, may be
utilized in assays for the evaluation of compounds for their ability to inhibit enzymatic

activation of the carotenoid biosynthetic enzymes disclosed herein. Assays may be conducted under well known experimental conditions which permit optimal enzymatic activity. For example, assays for phytoene synthase are presented by Neudert U. et al. (1998) *Biochim. Biophys. Acta* 1392:51-58. Assays for zeaxanthin epoxidase are presented

5 by Bouvier F. et al. (1996) *J. Biol. Chem.* 271:28861-28867).

CLAIMS

What is claimed is:

1. An isolated nucleic acid fragment encoding all or a substantial portion of a phytoene synthase comprising a member selected from the group consisting of:
 - 5 (a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14 and SEQ ID NO:16;
 - 10 (b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14 and SEQ ID NO:16; and
 - 15 (c) an isolated nucleic acid fragment that is complementary to (a) or (b).
2. The isolated nucleic acid fragment of Claim 1 wherein the nucleotide sequence of the fragment comprises all or a portion of the sequence set forth in a member selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7,
 - 20 SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13 and SEQ ID NO:15.
3. A chimeric gene comprising the nucleic acid fragment of Claim 1 operably linked to suitable regulatory sequences.
4. A transformed host cell comprising the chimeric gene of Claim 3.
5. A phytoene synthase polypeptide comprising all or a substantial portion of the
 - 25 amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14 and SEQ ID NO:16.
6. An isolated nucleic acid fragment encoding all or a substantial portion of a zeaxanthin epoxidase comprising a member selected from the group consisting of:
 - 30 (a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26;
 - 35 (b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26; and
 - (c) an isolated nucleic acid fragment that is complementary to (a) or (b).

7. The isolated nucleic acid fragment of Claim 6 wherein the nucleotide sequence of the fragment comprises all or a portion of the sequence set forth in a member selected from the group consisting of SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23 and SEQ ID NO:25.
- 5 8. A chimeric gene comprising the nucleic acid fragment of Claim 6 operably linked to suitable regulatory sequences.
9. A transformed host cell comprising the chimeric gene of Claim 8.
- 10 10. A zeaxanthin epoxidase polypeptide comprising all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26.
11. A method of altering the level of expression of a carotenoid biosynthetic enzyme in a host cell comprising:
- (a) transforming a host cell with the chimeric gene of any of Claims 3 and 8; and
- 15 (b) growing the transformed host cell produced in step (a) under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of altered levels of a carotenoid biosynthetic enzyme in the transformed host cell.
12. A method of obtaining a nucleic acid fragment encoding all or a substantial portion of the amino acid sequence encoding a carotenoid biosynthetic enzyme comprising:
- (a) probing a cDNA or genomic library with the nucleic acid fragment of any of Claims 1 and 6;
- (b) identifying a DNA clone that hybridizes with the nucleic acid fragment of any of Claims 1 and 6;
- 25 (c) isolating the DNA clone identified in step (b); and
- (d) sequencing the cDNA or genomic fragment that comprises the clone isolated in step (c)
- wherein the sequenced nucleic acid fragment encodes all or a substantial portion of the amino acid sequence encoding a carotenoid biosynthetic enzyme.
- 30 13. A method of obtaining a nucleic acid fragment encoding a substantial portion of an amino acid sequence encoding a carotenoid biosynthetic enzyme comprising:
- (a) synthesizing an oligonucleotide primer corresponding to a portion of the sequence set forth in any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25; and
- 35 (b) amplifying a cDNA insert present in a cloning vector using the oligonucleotide primer of step (a) and a primer representing sequences of the cloning vector
- wherein the amplified nucleic acid fragment encodes a substantial portion of an amino acid sequence encoding a carotenoid biosynthetic enzyme.

14. The product of the method of Claim 12.
15. The product of the method of Claim 13.
16. A method for evaluating at least one compound for its ability to inhibit the activity of a carotenoid biosynthetic enzyme, the method comprising the steps of:
 - 5 (a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a carotenoid biosynthetic enzyme, operably linked to suitable regulatory sequences;
 - (b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric
10 gene results in production of the carotenoid biosynthetic enzyme encoded by the operably linked nucleic acid fragment in the transformed host cell;
 - (c) optionally purifying the carotenoid biosynthetic enzyme expressed by the transformed host cell;
 - 15 (d) treating the carotenoid biosynthetic enzyme with a compound to be tested; and
 - (e) comparing the activity of the carotenoid biosynthetic enzyme that has been treated with a test compound to the activity of an untreated carotenoid biosynthetic enzyme,
 - 20 thereby selecting compounds with potential for inhibitory activity.

FIGURE 1

```

SEQ ID NO: 27
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SEQ ID NO: 28      SPAYSSLPVPAGEAVSSEQVYDVVLKQA-ALLKRQLRTPVLD---ARPDMDMP--
SEQ ID NO: 02      DELVDGPNASYITPTALDRWEKRLDLFEGRPYDMDAALSDTVSKFPVDIQPFKDMVQG
SEQ ID NO: 14      GTSAFSS---AVAAATETSRSEERVYEVVLKQA-ALVKEHKRGTKIALDLDKDVEADFN--
                                     61      120

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| | |
|---------------|--|
| SEQ ID NO: 27 | --LGLLSEAYDRCGEVCAYAKTFYL-GTMLMTPDRRRRAIWAIYVWCRRTDELVDGPNAS |
| SEQ ID NO: 28 | -RNLG-KEYDRCGEICEEYAKTFYL-GTMLMTEERRRAIWAIYVWCRRTDELVDGPNAN |
| SEQ ID NO: 02 | MRLDLWKSRYMTFDEL---YLICYVAGTQLMTPERRKAVWAIYVWCRRTDELVDGPNAS |
| SEQ ID NO: 14 | -NVDLLNAAAYDRCGEVCAYAKTFYL-GTQLMTAERRKAIWAIYVWCRRTDELVDGPNAS |
| | 121 |
| | 180 |

| | |
|---------------|--|
| SEQ ID NO: 27 | HTPQALDRWEARLEDIFNGRPFEDMLDAALSDTVSRFPVDIQFFRDMVEGMRMDLWKSRY |
| SEQ ID NO: 28 | YITPTALDRWEKRLDLFTGRPYDMLDAALSDTISRFPIDIQFFRDMIEGMRSDLRKTRY |
| SEQ ID NO: 02 | YITPTALDRWEKRLDLFEGRPYDMDAALSDTVSKFPVDIQFFKDMVQGMRLDLWKSRY |
| SEQ ID NO: 14 | HITPGALDRWEQRLSDVFEGRPYDMDAALSHTVSKYPVDIQFFKDMIEGMRVDLRKSRY |
| | 181 |
| | 240 |

FIGURE 1 (CONTINUED)

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SEQ ID NO: 02 MTFDELYLYCYVAGTVGLMTVPVPMGIAPIESKATTESVYNAALALGIANQLTNILRDVGE
SEQ ID NO: 14 NNFDELYLYCYVAGTVGLMSVPVPMGIAPIESNASESIYNAALALGIANQLTNILRDVGE
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241

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SEQ ID NO: 28 DARRGRIYLPQDELAQAGLSDEDFKGVVTNRWRNFMKRQIKRARMFFEEAERGVELSQ
SEQ ID NO: 02 DARRGRIYLPQDELAQAGLSEEDIFRGKVTGKWRRFMKGQIQARLFFDEAEKGVTHLDS
SEQ ID NO: 14 DARRGRVYLPQDELAQAGLSDDDFRGRVTDKWRKFMKGQIKRARMFFDEAERGVAELNS
301
360

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SEQ ID NO: 28 ASRWPVWASLLLYRQILDEIEANDYNNFTKRAYVGKPKLLALPVAYGKSLLP-----CS
SEQ ID NO: 02 ASRWPVLASLLLYRQILDAIEANDYNNFTKRAYVGKAKKLLSLPLAYARAAVAP.-----
SEQ ID NO: 14 ASRWPVWASLLLYRQILDSIEANDYNNFTKRAYVGKVKKLLSLPTAYGFSLLGPQKFTKM
361
420

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SEQ ID NO: 28 LRNGQT
SEQ ID NO: 02 -----
SEQ ID NO: 14 VR--R.
421 426

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<130> BB-1115-B

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 Ser Lys Phe Pro Val Asp Ile Gln Pro Phe Lys Asp Met Val Gln Gly
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 260 265 270
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 35 40 45
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 Glu Gly Met Arg Met Asp Leu Lys Lys Ser Arg Tyr Arg Ser Phe Asp
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gaggagtatg ccaagacctt ttaccttga actatgctca tgacggagga ccgacggngc 660
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          35              40              45

Thr Pro Ala Gly Glu Ala Val Ile Ser Ser Glu Gln Lys Val Tyr Asp
          50              55              60

Val Val Leu Lys Gln Ala Ala Leu Leu Lys Arg His Leu Arg Pro Gln
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Pro His Thr Ile Pro Ile Val Pro Lys Asp Leu Asp Leu Pro Arg Asn
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Ala Lys Thr Phe Tyr Leu Gly Thr Met Leu Met Thr Glu Asp Arg Arg
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 Arg Ser Phe Asp Glu Leu Tyr Leu Tyr Cys Tyr Tyr Val Ala Gly Thr
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Glu Gly Met Arg Ser Asp Leu Arg Lys Thr Arg Tyr Lys Asn Phe Asp
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Glu Leu Tyr Met Tyr Cys Tyr Tyr Val Ala Gly Thr Val Gly Leu Met
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Ser Val Pro Val Met Gly Ile Ala Pro Glu Ser Lys Ala Thr Thr Glu
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Ser Val Tyr Ser Ala Ala Leu Ala Leu Gly Asn Ala Asn Gln Leu Thr
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115 120 125

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 Ile Lys Arg Ala Arg Met Phe Phe Glu Glu Ala Glu Arg Gly Val Thr
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 Tyr Arg Gln Ile Leu Asp Glu Ile Glu Ala Asn Asp Tyr Asn Asn Phe
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Gln Lys

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 Met Leu Asp Ala Ala Leu Ser Asp Thr Val Ala Lys Phe Pro Val Asp
 35 40 45
 Ile Gln Pro Phe Lys Asp Met Ile Glu Gly Met Arg Leu Asp Leu Lys
 50 55 60
 Lys Pro Arg Tyr Arg Asn Phe Asp Glu Leu Tyr Leu Tyr Cys Tyr Tyr
 65 70 75 80
 Val Ala Gly Thr Val Gly Ile Met Ser Val Pro Ile Met Gly Ile Ser
 85 90 95
 Pro Asn Ser Gln Ala Thr Thr Glu Ser Val Tyr Asn Ala Ala Leu Ala
 100 105 110
 Leu Gly Ile Ala Asn Gln Leu Thr Asn Ile Leu Arg Asp Val Gly Glu
 115 120 125
 Asp Ala Ser Arg Gly Arg Val Tyr Leu Pro Gln Asp Glu Leu Ala Gln
 130 135 140
 Ala Gly Leu Ser Asp Glu Asp Ile Phe Ala Gly Lys Val Thr Asp Lys
 145 150 155 160
 Trp Arg Asn Phe Met Lys Ser Gln Ile Lys Arg Ala Arg Met Phe Phe
 165 170 175
 Asp Glu Ala Glu Lys Gly Val Thr Glu Leu Asn Glu Ala Ser Arg Trp
 180 185 190
 Pro Val Trp Ala Ser Leu Leu Leu Tyr Arg Gln Ile Leu Asp Glu Ile
 195 200 205
 Glu Ala Asn Asp Tyr Asn Asn Phe Thr Arg Arg Ala Tyr Val Ser Lys
 210 215 220
 Ala Lys Lys Leu Leu Ser Leu Pro Ala Ala Tyr Ala Arg Ser Met Val
 225 230 235 240
 Pro Pro Ser Lys Lys Leu Ser Ser Val Met Lys Thr
 245 250

<210> 13

<211> 1397

<212> DNA

<213> Glycine max

<400> 13

gtttttgctaa cacaagtata cactcattct caaaagggtt tcatccaatt tctttccctc 60
 tcttttcatt ggtgtgcact ttcacttggt gagctgcac aactgcagt gaaattgtgc 120

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tttgttcttg agatgtcttg tgttcttctt tgggtgagtt gtggacccaa agagaacatc 180
aactccttgg tgagtttttc atgcaggagt agtagtggtg gtgaaagaac acaaaagaga 240
ttttctggaa tcagtttttc tagtggtact tctgcttttt caagtgcagt ggcagctact 300
gagacttcaa gatcttcaga ggagagggtc tatgaagtgg ttctgaagca agcagctttg 360
gtaaaagaac acaaaagggg tacaaaaata gctttggatt tggacaaaaga tgttgaggct 420
gatttcaaca atgtggatct gttgaatgctg gcttatgatc ggtgtggtga agtttgtgct 480
gagtatgcc aagacatttta cttaggcaca caattgatga ctgcagagcg ccgaaaagca 540
at ttggggcaa tttatgtgtg gtgcagaaga actgatgagc tagtggtggtg cccaaatgct 600
tcacacatca cccctggggc cttggacagg tgggagcaac gattgagtga tgtttttgaa 660
ggtcgaccct atgatatgta tgatgctgcc ctctcacata ctgtctcaaa gtacccggtt 720
gatattcagc ctttcaagga catgatcgaa gggatgaggg tggacctgag aaagtcaaga 780
tacaataact ttgatgagct ctacctttac tgctactatg ttgctgggac agtaggcctt 840
atgagtgtcc cagtaatggg gatagcacca gaatcaaatg cttcatcaga ggcatttat 900
aatgctgcac tggctctagg cattgcaaat caacttacca acatacttag agatgttgga 960
gaagatgcta gaagaggaag agtatatctc ccacaagatg aattggcaca agctggcctt 1020
tcagatgatg acattttccg cggaagagtt acagacaaat ggcggaaatt catgaaggga 1080
caaataaaga gggcgaggat gttttttgat gaggcagaga gagggggttc agagctcaac 1140
tcagctagca ggtggcctgt gtgggcatca ttgttgtgtg ataggcaaat attagattcc 1200
attgaagcca atgattataa taacttcaca aaaaggcat atgtaggaaa agtaaaagaaa 1260
ctcttgtcac tactactgct ctatggtttt tcacttctag gccctcagaa gtttaccaaa 1320
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tcaagttaaa aaaaaaa 1397

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<210> 14
 <211> 400
 <212> PRT
 <213> Glycine max

<400> 14
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 Asn Ser Leu Val Ser Phe Ser Cys Arg Ser Ser Ser Gly Gly Glu Arg
 20 25 30
 Thr Gln Lys Arg Phe Ser Gly Ile Ser Phe Ala Ser Gly Thr Ser Ala
 35 40 45
 Phe Ser Ser Ala Val Ala Ala Thr Glu Thr Ser Arg Ser Ser Glu Glu
 50 55 60
 Arg Val Tyr Glu Val Val Leu Lys Gln Ala Ala Leu Val Lys Glu His
 65 70 75 80
 Lys Arg Gly Thr Lys Ile Ala Leu Asp Leu Asp Lys Asp Val Glu Ala
 85 90 95
 Asp Phe Asn Asn Val Asp Leu Leu Asn Ala Ala Tyr Asp Arg Cys Gly
 100 105 110
 Glu Val Cys Ala Glu Tyr Ala Lys Thr Phe Tyr Leu Gly Thr Gln Leu
 115 120 125
 Met Thr Ala Glu Arg Arg Lys Ala Ile Trp Ala Ile Tyr Val Trp Cys
 130 135 140
 Arg Arg Thr Asp Glu Leu Val Asp Gly Pro Asn Ala Ser His Ile Thr
 145 150 155 160
 Pro Gly Ala Leu Asp Arg Trp Glu Gln Arg Leu Ser Asp Val Phe Glu
 165 170 175

Gly Arg Pro Tyr Asp Met Tyr Asp Ala Ala Leu Ser His Thr Val Ser
 180 185 190
 Lys Tyr Pro Val Asp Ile Gln Pro Phe Lys Asp Met Ile Glu Gly Met
 195 200 205
 Arg Val Asp Leu Arg Lys Ser Arg Tyr Asn Asn Phe Asp Glu Leu Tyr
 210 215 220
 Leu Tyr Cys Tyr Tyr Val Ala Gly Thr Val Gly Leu Met Ser Val Pro
 225 230 235 240
 Val Met Gly Ile Ala Pro Glu Ser Asn Ala Ser Ser Glu Ser Ile Tyr
 245 250 255
 Asn Ala Ala Leu Ala Leu Gly Ile Ala Asn Gln Leu Thr Asn Ile Leu
 260 265 270
 Arg Asp Val Gly Glu Asp Ala Arg Arg Gly Arg Val Tyr Leu Pro Gln
 275 280 285
 Asp Glu Leu Ala Gln Ala Gly Leu Ser Asp Asp Asp Ile Phe Arg Gly
 290 295 300
 Arg Val Thr Asp Lys Trp Arg Lys Phe Met Lys Gly Gln Ile Lys Arg
 305 310 315 320
 Ala Arg Met Phe Phe Asp Glu Ala Glu Arg Gly Val Ala Glu Leu Asn
 325 330 335
 Ser Ala Ser Arg Trp Pro Val Trp Ala Ser Leu Leu Leu Tyr Arg Gln
 340 345 350
 Ile Leu Asp Ser Ile Glu Ala Asn Asp Tyr Asn Asn Phe Thr Lys Arg
 355 360 365
 Ala Tyr Val Gly Lys Val Lys Lys Leu Leu Ser Leu Pro Thr Ala Tyr
 370 375 380
 Gly Phe Ser Leu Leu Gly Pro Gln Lys Phe Thr Lys Met Val Arg Arg
 385 390 395 400

<210> 15
 <211> 1021
 <212> DNA
 <213> Triticum aestivum

<400> 15
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 gcgctcgatc ggtgggagaa gagattagag gatctcttcg aaggccgccc atatgatatg 120
 tatgatgcag ccctctcaga tacagcgtca aagtttccaa ttgatatcca gccattcaga 180
 gacatgattg aagggatgag gctcgacctt tggaaatcga ggtataggac ctttgacgag 240
 ctctacctct actgctacta cgtcgctggc actgtcggtc tcatgacggt accggtgatg 300
 gggattgctc cggactcaaa ggctcagca gagagcgtgt acaatgccgc actggccctt 360
 ggcattgccca accagctcac aaacatcctc cgagacgtag gagaagactc aagaaggggg 420
 agaataacc ttccactgga cgaactggca caggcgggtc tgacagaaga ggacatattc 480
 agagggaag tgacggataa atggaggagg ttcataagg ggcaaatcca gcgcgccagg 540
 ctcttctttg acgaggccga gaaggcgctc atgcatctag actccgcgag cagatggccg 600
 gtcctggcat cgctgtggct gtacaggcag atcctggacg ccatcgaggc caacgactac 660
 aacaacttca ccaagcgcgc gtacgtgggc aaggcaaga agttctgtc tctaccggcc 720

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gcgtacgcga gggcggtctt ctgcgcatga gcaaagcaat cccgtagatc agatgttttt 780
tcttcttctt tttcttctt tttgtcctgt caccctacaa tgatttttgt tggctgttgt 840
atatactcag ctatatgttt gccatacgcc cgccgcggta tttaggtcaa gggaccgacg 900
tcgggccccg ctgtactgaa gtctgaaaca cttgttgta ccacacagtg gagaatcaaa 960
attgctccag ttgaatgaag aagaaacaaa cactctttct tcctaaaaaa aaaaaaaaaa 1020
a 1021

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<210> 16
 <211> 248
 <212> PRT
 <213> Triticum aestivum

<400> 16
 Thr Arg Arg Thr Asp Glu Leu Val Asp Gly Pro Asn Ser Ser Tyr Ile
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 Thr Pro Lys Ala Leu Asp Arg Trp Glu Lys Arg Leu Glu Asp Leu Phe
 20 25 30
 Glu Gly Arg Pro Tyr Asp Met Tyr Asp Ala Ala Leu Ser Asp Thr Ala
 35 40 45
 Ser Lys Phe Pro Ile Asp Ile Gln Pro Phe Arg Asp Met Ile Glu Gly
 50 55 60
 Met Arg Leu Asp Leu Trp Lys Ser Arg Tyr Arg Thr Phe Asp Glu Leu
 65 70 75 80
 Tyr Leu Tyr Cys Tyr Tyr Val Ala Gly Thr Val Gly Leu Met Thr Val
 85 90 95
 Pro Val Met Gly Ile Ala Pro Asp Ser Lys Ala Ser Ala Glu Ser Val
 100 105 110
 Tyr Asn Ala Ala Leu Ala Leu Gly Ile Ala Asn Gln Leu Thr Asn Ile
 115 120 125
 Leu Arg Asp Val Gly Glu Asp Ser Arg Arg Gly Arg Ile Tyr Leu Pro
 130 135 140
 Leu Asp Glu Leu Ala Gln Ala Gly Leu Thr Glu Glu Asp Ile Phe Arg
 145 150 155 160
 Gly Lys Val Thr Asp Lys Trp Arg Arg Phe Met Lys Gly Gln Ile Gln
 165 170 175
 Arg Ala Arg Leu Phe Phe Asp Glu Ala Glu Lys Gly Val Met His Leu
 180 185 190
 Asp Ser Ala Ser Arg Trp Pro Val Leu Ala Ser Leu Trp Leu Tyr Arg
 195 200 205
 Gln Ile Leu Asp Ala Ile Glu Ala Asn Asp Tyr Asn Asn Phe Thr Lys
 210 215 220
 Arg Ala Tyr Val Gly Lys Ala Lys Lys Phe Leu Ser Leu Pro Ala Ala
 225 230 235 240
 Tyr Ala Arg Ala Ala Leu Ser Pro
 245

<210> 17
<211> 722
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (324)

<220>
<221> unsure
<222> (525)

<220>
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<220>

<221> unsure

<222> (719)

<400> 17

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gtcaacggca tcgttgacgg cgtttctggc tcctgggtaca tcaagtttga tacgtttact 120
cctgcagctg agcgggggct cccggtcaca agggtcatta gccgcatgac gctgcaacag 180
atccttgctc gagcagttgg cgatgacgct atattgaatg gaagccatgt agtcgatttt 240
acagatgatg gcagtaaggt tactgccata ttggaggacg gtaggatatt tgaaggtgac 300
cttttggttg gtgccgatgg aatntggcca aaggtaggga agacactatt cggggcactca 360
gatgccacct attcaggtta catctgcaat tccagtgtag cagattttgt gccacctgat 420
atcgatacag ttgggtaccg agtatttctt ggccacaaac agtacttcgt ctcttcggat 480
gtcgggtgctg gtaaaatgca atggtagcgt ttccacaatg aagangctgg tngnactgnc 540
cctgaaatgg caanaaagaa aaaattgctt gagatattcg acggntgggt ggataatggt 600
aatgatttga tanatgcaac tgaggaagaa gcagntcttc gncngatat ntacggcggc 660
ccacctaanc gatgnnattg gggggaaagg ccgggcacct tgcttgggga tctggccang 720
ct

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<210> 18

<211> 121

<212> PRT

<213> Zea mays

<220>

<221> UNSURE

<222> (95)

<400> 18

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Gly Cys Val Thr Gly Asp Arg Val Asn Gly Ile Val Asp Gly Val Ser
 1           5           10           15

Gly Ser Trp Tyr Ile Lys Phe Asp Thr Phe Thr Pro Ala Ala Glu Arg
 20           25           30

Gly Leu Pro Val Thr Arg Val Ile Ser Arg Met Thr Leu Gln Gln Ile
 35           40           45

Leu Ala Arg Ala Val Gly Asp Asp Ala Ile Leu Asn Gly Ser His Val
 50           55           60

Val Asp Phe Thr Asp Asp Gly Ser Lys Val Thr Ala Ile Leu Glu Asp
 65           70           75           80

Gly Arg Ile Phe Glu Gly Asp Leu Leu Val Gly Ala Asp Gly Xaa Trp
 85           90           95

Ser Lys Val Arg Lys Thr Leu Phe Gly His Ser Asp Ala Thr Tyr Ser
100           105           110

Gly Tyr Ile Cys Asn Ser Ser Val Ala
115           120

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<210> 19

<211> 1246

<212> DNA

<213> Zea mays

<220>

<221> unsure

<222> (367)

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<400> 19
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ccagctggct gtagagctag agaatgcctg gcaagagagt gtcaaaactg aaactcctat 180
agacatagtt tcctccttga ggcgctacga gaaagagaga aggctgcgtg ttgctattat 240
acatggactg gcaagaatgg cagcaatcat ggctaccacc tatagaccgt acttgggtgt 300
tggtctaggg cctttatcgt ttttgaccaa gttgcggata ccacaccctg gaagagtcgg 360
tggcagnttc ttcatcaagt atggaatgcc tacgatgttg agctgggtgc ttggtggcaa 420
cagctcaaaa ctagaaggaa gacttttaag ctgccgactt tctgacaagg caaatgacca 480
gctttatcaa tggtttgagg atgatgacgc actggaagaa gctatgggtg gagaatggta 540
cctcatcgca acaagtgaag gaaactgcaa tagcttgcaag ccatttcatt taattagggg 600
tgagcagagg tcaactcttg ttggaagccg gtcagatcct aatgattcag cttcttccct 660
atcattgtcc tctccacaga tatcagaaag acatgctact atcacatgca agaataaagc 720
tttctatctg actgatctcg gaagcgaaca tggtagcttg attaccgaca atgaaggtag 780
acgttaccgc gtgccaccaa acttcccagt tcgtttccat ccctccgatg tcattgagtt 840
tggttccgat aagaaggcta tggtccgggt gaaggtgctg aacacgctcc cgtatgaatc 900
tgcaagaagt gggaatcggc agcaacagca agtccttcag gcagcatgaa tggagacact 960
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tgcatacagg aaaggataca ctcgttctcg aatatttgct gtcgtgtagt tcaattttaa 1080
actaaaacgt gacaaatgaa aaaacgaagg aagtagaaga tatgtcaaaa cacatgcaat 1140
ttttgcatcc atgaagatgc caaacaggat cttgaatact agcacctagc ggattgaaat 1200
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<210> 20
<211> 315
<212> PRT
<213> Zea mays

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<220>
<221> UNSURE
<222> (7)

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<220>
<221> UNSURE
<222> (122)

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<400> 20
Arg Lys Arg Ser Ser Asp Xaa Ala Glu Arg His Arg Ser Val Ser Leu
 1             5             10             15

Leu Asn Ser Arg Ser Leu Ala Arg Ser Lys Arg Lys Lys Leu Ala Phe
      20             25             30

Ser Met Ala Ile Glu Asp Gly Tyr Gln Leu Ala Val Glu Leu Glu Asn
      35             40             45

Ala Trp Gln Glu Ser Val Lys Thr Glu Thr Pro Ile Asp Ile Val Ser
      50             55             60

Ser Leu Arg Arg Tyr Glu Lys Glu Arg Arg Leu Arg Val Ala Ile Ile
      65             70             75             80

His Gly Leu Ala Arg Met Ala Ala Ile Met Ala Thr Thr Tyr Arg Pro
      85             90             95

Tyr Leu Gly Val Gly Leu Gly Pro Leu Ser Phe Leu Thr Lys Leu Arg
      100            105            110

Ile Pro His Pro Gly Arg Val Gly Gly Xaa Phe Phe Ile Lys Tyr Gly
      115            120            125

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Met Pro Thr Met Leu Ser Trp Val Leu Gly Gly Asn Ser Ser Lys Leu
 130 135 140

Glu Gly Arg Leu Leu Ser Cys Arg Leu Ser Asp Lys Ala Asn Asp Gln
 145 150 155 160

Leu Tyr Gln Trp Phe Glu Asp Asp Asp Ala Leu Glu Glu Ala Met Gly
 165 170 175

Gly Glu Trp Tyr Leu Ile Ala Thr Ser Glu Gly Asn Cys Asn Ser Leu
 180 185 190

Gln Pro Ile His Leu Ile Arg Asp Glu Gln Arg Ser Leu Phe Val Gly
 195 200 205

Ser Arg Ser Asp Pro Asn Asp Ser Ala Ser Ser Leu Ser Leu Ser Ser
 210 215 220

Pro Gln Ile Ser Glu Arg His Ala Thr Ile Thr Cys Lys Asn Lys Ala
 225 230 235 240

Phe Tyr Leu Thr Asp Leu Gly Ser Glu His Gly Thr Trp Ile Thr Asp
 245 250 255

Asn Glu Gly Arg Arg Tyr Arg Val Pro Pro Asn Phe Pro Val Arg Phe
 260 265 270

His Pro Ser Asp Val Ile Glu Phe Gly Ser Asp Lys Lys Ala Met Phe
 275 280 285

Arg Val Lys Val Leu Asn Thr Leu Pro Tyr Glu Ser Ala Arg Ser Gly
 290 295 300

Asn Arg Gln Gln Gln Gln Val Leu Gln Ala Ala
 305 310 315

<210> 21
 <211> 926
 <212> DNA
 <213> Glycine max

<400> 21
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 aaaactcttt gggcagcaag aagcaaatta ctgggtttc acatgctaca gtggattaac 120
 aagctatgtg ccccatata ttgataccgt tgggtatcgg gtgttcttgg gcttgaacca 180
 gtactttggt gcttcagatg ttggccatgg gaagatgcag tggatgctt tccatgggga 240
 accccttca agtgaccctt tcccagaagg taagaagaag aggcttttgg atctctttgg 300
 taattggtgc gatgaagtga ttgactcat atcagaaaca ccagaacata tgattataca 360
 gagggatata tatgacagag acatgatcaa cacttgggga attgggagag tgactttggt 420
 aggtgatgca gcacatccaa tgcaaccaa tcttgggtcaa ggagggtgta tggcaataga 480
 ggattgttac caactgatac ttgagctaga caagggtgct aaacatggct ctgacgggtc 540
 tgaagtattc tcagctctta gaagatatga gaagaaaaga atcccccgag ttaggggtgtt 600
 acacacagct agcaggatgg catcgcaaat gttagtcaac taccggcctt atattgaatt 660
 taaattttgg cctctatcaa atgtaacaac tatgcagata aagcaccctg gcattcatgt 720
 agctcaagcc cttttcaagt tcacttttcc acaatttgtt acttggatga ttgctggcca 780
 tgggtgtgtg tgaacactca tgcaacttga aaataaaaag ggctcaacaa ttttaacatg 840
 atggtagtta aaagttaatt ttattgggct atgtaggaaac ttttctttcg gaataaacgt 900
 gccataattt aaaaaaaaaa aaaaaa 926

<210> 22
 <211> 263
 <212> PRT
 <213> Glycine max

<400> 22
 His Glu His Asp Gly Asp Ile Leu Ile Gly Ala Asp Gly Ile Trp Ser
 1 5 10 15
 Glu Val Arg Ser Lys Leu Phe Gly Gln Gln Glu Ala Asn Tyr Ser Gly
 20 25 30
 Phe Thr Cys Tyr Ser Gly Leu Thr Ser Tyr Val Pro Pro Tyr Ile Asp
 35 40 45
 Thr Val Gly Tyr Arg Val Phe Leu Gly Leu Asn Gln Tyr Phe Val Ala
 50 55 60
 Ser Asp Val Gly His Gly Lys Met Gln Trp Tyr Ala Phe His Gly Glu
 65 70 75 80
 Pro Pro Ser Ser Asp Pro Phe Pro Glu Gly Lys Lys Lys Arg Leu Leu
 85 90 95
 Asp Leu Phe Gly Asn Trp Cys Asp Glu Val Ile Ala Leu Ile Ser Glu
 100 105 110
 Thr Pro Glu His Met Ile Ile Gln Arg Asp Ile Tyr Asp Arg Asp Met
 115 120 125
 Ile Asn Thr Trp Gly Ile Gly Arg Val Thr Leu Leu Gly Asp Ala Ala
 130 135 140
 His Pro Met Gln Pro Asn Leu Gly Gln Gly Gly Cys Met Ala Ile Glu
 145 150 155 160
 Asp Cys Tyr Gln Leu Ile Leu Glu Leu Asp Lys Val Ala Lys His Gly
 165 170 175
 Ser Asp Gly Ser Glu Val Ile Ser Ala Leu Arg Arg Tyr Glu Lys Lys
 180 185 190
 Arg Ile Pro Arg Val Arg Val Leu His Thr Ala Ser Arg Met Ala Ser
 195 200 205
 Gln Met Leu Val Asn Tyr Arg Pro Tyr Ile Glu Phe Lys Phe Trp Pro
 210 215 220
 Leu Ser Asn Val Thr Thr Met Gln Ile Lys His Pro Gly Ile His Val
 225 230 235 240
 Ala Gln Ala Leu Phe Lys Phe Thr Phe Pro Gln Phe Val Thr Trp Met
 245 250 255
 Ile Ala Gly His Gly Leu Trp
 260

<210> 23
 <211> 1528
 <212> DNA
 <213> Glycine max

<400> 23
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 acaattctct taacccttca acaaccgttt tctcaagaac ccatttctca gttcccttga 120
 ataaagagct tccactggat gcttcacctt ttgttgttgg ctataactgt ggtgtaggat 180
 gcagaacaag gaagcaaagg aagaaagtga tgcattgtgaa gtgtgcagtgt gtggaggctc 240
 caccaggtgt ttcaccctca gcaaaagatg ggaatgggaa ccacccttc cgaagaagca 300
 gcttcgtata cttgtggctg gtggagggat tggaggggtt gtttttgctt tgggctgcaa 360
 agagaaagggt gtttgagggt atggtgtttg agaaggactt gactgctata agaggggagg 420
 gacagtatag ggtccaatt cagattcaga gcaatgctt ggctgctttg gaagctattg 480
 attcagaggt tgctgatgaa gttatgagag ttggttgcatt cactggtgat agaatacatg 540
 gactttaga tggtgtttct ggttcttggg acgtcaagt tgcatacttc actcctgcag 600
 tggaacgtgg gcttcctgtc acaagagtta ttagtcgaat gggttttaca gagatccttg 660
 ctgcgcaggt tggggaagat atcattatga atgccaagta tggtgttaatt ttgtggatg 720
 atggaacaa ggttaacagta gagctagaga atggtcagaa atatgaagga gatgtcttgg 780
 ttggagcgga tgggaatatg tccaagggtg ggaagcagtt atttgggctc acagaagctg 840
 ttactctgg ttatacttgg tatactggca ttgcagattt tgtgcctgct gacattgaaa 900
 ctgttgata ccgagtattc ttgggacaca aacaatactt tgtatcttca gatgttgggt 960
 cgggaaagat gcaatggtat gcatctcaca aagaaactcc cgggtggggtt gatgagccca 1020
 acggaaaaaa ggaaagggtt cttaggatat ttgagggtct gtgtgaaagt gctgtagatc 1080
 tgatacttgc cacagaagaa gaagcaattc taagacgaga catatatgac aggataccaa 1140
 cattgacatg gggaaagggt cgcgtgactt tgcttgggtg ttccgtccat gccatgcagc 1200
 caaatatggg ccaaggagggt tgcattggct ttgaggacag ttatcaactt gcatgggagt 1260
 tggagaatgc atgggaacaa agtattaaat cagggaagtcc aattgacatt gattcttccc 1320
 taaggagcta cgagagagaa agaagactac gagttgccat tattcatgga atggctagaa 1380
 tggcggtctc catggcttcc acttacaagg catatctggg tggttggctt ggccctttag 1440
 aatttttgac taagtctcgt ataccacatc ctggaagagt tggaggaagg ttttttgtt 1500
 acatcatgat gccttctatg ttgatgtt 1528

<210> 24
 <211> 495
 <212> PRT
 <213> Glycine max

<400> 24
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 Phe Ser Arg Thr His Phe Ser Val Pro Leu Asn Lys Glu Leu Pro Leu
 20 25 30
 Asp Ala Ser Pro Phe Val Val Gly Tyr Asn Cys Gly Val Gly Cys Arg
 35 40 45
 Thr Arg Lys Gln Arg Lys Lys Val Met His Val Lys Cys Ala Val Val
 50 55 60
 Glu Ala Pro Pro Gly Val Ser Pro Ser Ala Lys Asp Gly Asn Gly Asn
 65 70 75 80
 His Pro Phe Arg Arg Ser Ser Phe Val Tyr Leu Trp Leu Val Glu Gly
 85 90 95
 Leu Glu Gly Trp Phe Leu Leu Trp Ala Ala Lys Arg Lys Gly Phe Glu
 100 105 110
 Val Met Val Phe Glu Lys Asp Leu Ser Ala Ile Arg Gly Glu Gly Gln
 115 120 125
 Tyr Arg Gly Pro Ile Gln Ile Gln Ser Asn Ala Leu Ala Ala Leu Glu
 130 135 140

Ala Ile Asp Ser Glu Val Ala Asp Glu Val Met Arg Val Gly Cys Ile
 145 150 155 160
 Thr Gly Asp Arg Ile Asn Gly Leu Val Asp Gly Val Ser Gly Ser Trp
 165 170 175
 Tyr Val Lys Phe Asp Thr Phe Thr Pro Ala Val Glu Arg Gly Leu Pro
 180 185 190
 Val Thr Arg Val Ile Ser Arg Met Val Leu Gln Glu Ile Leu Ala Arg
 195 200 205
 Ala Val Gly Glu Asp Ile Ile Met Asn Ala Ser Asn Val Val Asn Phe
 210 215 220
 Val Asp Asp Gly Asn Lys Val Thr Val Glu Leu Glu Asn Gly Gln Lys
 225 230 235 240
 Tyr Glu Gly Asp Val Leu Val Gly Ala Asp Gly Ile Trp Ser Lys Val
 245 250 255
 Arg Lys Gln Leu Phe Gly Leu Thr Glu Ala Val Tyr Ser Gly Tyr Thr
 260 265 270
 Cys Tyr Thr Gly Ile Ala Asp Phe Val Pro Ala Asp Ile Glu Thr Val
 275 280 285
 Gly Tyr Arg Val Phe Leu Gly His Lys Gln Tyr Phe Val Ser Ser Asp
 290 295 300
 Val Gly Ala Gly Lys Met Gln Trp Tyr Ala Phe His Lys Glu Thr Pro
 305 310 315 320
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 Leu Pro Cys Ser Leu Arg Asn Gly Gln Thr
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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|---|--|--|---|
| (51) International Patent Classification ⁶ : C12N 15/82, 9/02, 5/10, G01N 33/50 // A01H 5/00 | | A3 | (11) International Publication Number: WO 99/55889 |
| | | | (43) International Publication Date: 4 November 1999 (04.11.99) |
| (21) International Application Number: PCT/US99/08789 | | (81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). | |
| (22) International Filing Date: 22 April 1999 (22.04.99) | | Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. | |
| (30) Priority Data: 60/083,042 24 April 1998 (24.04.98) US | | (88) Date of publication of the international search report: 6 April 2000 (06.04.00) | |
| (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). | | | |
| (72) Inventors; and (75) Inventors/Applicants (for US only): CAHOON, Rebecca, E. [US/US]; 2331 West 18th Street, Wilmington, DE 19806 (US). HITZ, William, D. [US/US]; 404 Hillside Road, Wilmington, DE 19807 (US). SHEN, Jennie, Bih-Jien [US/US]; 15 Bromley Court, Wilmington, DE 19810 (US). WILLIAMS, Mark, E. [US/US]; 37 Country Hills Drive, Newark, DE 19711 (US). | | | |
| (74) Agent: MAJARIAN, William, R.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US). | | | |
| (54) Title: CAROTENOID BIOSYNTHESIS ENZYMES | | | |
| (57) Abstract | | | |
| <p>This invention relates to an isolated nucleic acid fragment encoding a carotenoid biosynthetic enzyme. The invention also relates to the construction of a chimeric gene encoding all or a portion of the carotenoid biosynthetic enzyme, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the carotenoid biosynthetic enzyme in a transformed host cell.</p> | | | |
| <pre> SEQ ID NO:27 DDDIVLPGH----- SEQ ID NO:28 NAILLVRAASPL--SAADSISHQ-GTLQCSILLKTKRFAARRNMPCLLGLPWEAGRP SEQ ID NO:02 EEEEEVRLWGLGDAYDRGCEVCARYAKTFYLGTLMTFERRKAVN--AITW-CHRT SEQ ID NO:14 MSGVLLVSC-----GPKEMINSL-VSFCSRSSGGER-TQKRFSGISFAS----- 1 </pre> | | | |
| <pre> SEQ ID NO:27 SPAYISLFPVHPAGEAVVSEGVYDVVLNQA-ALLRGLATFVLD--KSPEDKMP-- SEQ ID NO:28 DELVDGPHASTITFIALDRWEKRLDLTGCPITDYDAALSDTVSKTFVDIQFEDKVOG SEQ ID NO:02 GTSAPFS--AAATFTRSSSEERVYEVVLNQA-ALVKEHGGTKIALDLKQVADTFH- 61 </pre> | | | |
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| <pre> * * * * * SEQ ID NO:27 ASRNPVLASILLTRKILDEIARDTNTFTTRATYVGRKLLSLPFIAYASLVPFSTSCP SEQ ID NO:28 ASRNPVLASILLTRKILDEIARDTNTFTTRATYVGRKLLSLPFIAYASLVPFSTSCP SEQ ID NO:02 ASRNPVLASILLTRKILDEIARDTNTFTTRATYVGRKLLSLPFIAYASLVPFSTSCP SEQ ID NO:14 ASRNPVLASILLTRKILDEIARDTNTFTTRATYVGRKLLSLPFIAYASLVPFSTSCP 361 </pre> | | | |
| <pre> SEQ ID NO:27 L--AKT SEQ ID NO:28 LRMGT SEQ ID NO:02 ----- SEQ ID NO:14 VR--R. 421 426 </pre> | | | |

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| DK | Denmark | LR | Liberia | SG | Singapore | | |
| EE | Estonia | | | | | | |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/08789

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N9/02 C12N5/10 G01N33/50 //A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| X | BAYSDORFER, C.: "the Maize cDNA program - unpublished" EMBL SEQUENCE DATA LIBRARY, 11 September 1996 (1996-09-11), XP002129006 heidelberg, germany accession no. AA051885 --- | 1-3 |
| X | YAMAMOTO, K. AND SASAKI, T.: "rice cDNA from panicle (longer than 10cm)" EMBL SEQUENCE DATA LIBRARY, 20 September 1997 (1997-09-20), XP002128998 heidelberg, germany accession no.C73801 --- -/-- | 1,2 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

2 February 2000

Date of mailing of the international search report

23 02 00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Holtorf, S

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/08789

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|---------------------------|
| X | YAMAMOTO, K. AND SASAKI, T.: "rice cDNA from panicle (between 3cm to 10cm)" EMBL SEQUENCE DATA LIBRARY, 21 September 1997 (1997-09-21), XP002128999 heidelberg, germany accession no. C74590 --- | 1,2 |
| X | DATABASE WPI Section Ch, Week 199824 Derwent Publications Ltd., London, GB; Class C06, AN 1998-264853 XP002129005 & JP 10 084966 A (IWATE KEN), 7 April 1998 (1998-04-07) abstract --- | 1-5,11 |
| X | CASTRIGNANO, F. AND GIULIANO, G.: "untitled" SWISSPROT SEQUENCE DATA LIBRARY, 1 January 1998 (1998-01-01), XP002129000 accession no. 022375 --- | 5 |
| X | BARTLEY, G.E. AND SCOLNIK, P.A.: "cDNA cloning, expression during development, and genome mapping of PSY2, a second tomato gene encoding phytoene synthase" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 34, 1993, pages 25718-25721, XP002112337 cited in the application the whole document --- | 1-4 |
| X | WO 97 46690 A (ZENECA LTD ;DRAKE CAROLINE RACHEL (GB); BIRD COLIN ROGER (GB); SCH) 11 December 1997 (1997-12-11) the whole document --- | 1-5,11 |
| Y | the whole document --- | 12-15 |
| X | WO 96 02650 A (ZENECA LTD ;KARVOUNI ZOI (GR); JOHN ISAAC (GB); TAYLOR JANE (GB);) 1 February 1996 (1996-02-01) example 1 --- | 1-5,11 |
| Y | example 1 --- | 12,14 |
| X | US 5 705 624 A (KUMAGAI MONTO HIROSHI ET AL) 6 January 1998 (1998-01-06) column 4, lines 54-61; page 5 + 6; example 1; claims --- | 1-5,11, 12,14 13,15 |
| Y | column 4, lines 54-61; page 5 + 6; example 1; claims --- | 13,15 |
| | --- | |
| | -/-- | |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/08789

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | BURKHARDT, P.K., ET AL.: "Transgenic rice (Oryza sativa) endosperm expressing daffodil (Narcissus pseudonarcissus) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis" THE PLANT JOURNAL, vol. 11, no. 5, 1997, pages 1071-1078, XP002087151 the whole document | 1-5,11 |
| X | & SCHLEDZ, M., ET AL.: "phytoene synthase from Narcissus pseudonarcissus: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering" EMBL SEQUENCE DATA LIBRARY, 18 April 1995 (1995-04-18), heidelberg, germany accession no. X78814 --- | 1-5 |
| X | SANDMANN, G., ET AL.: "a new non-radioactive assay of phytoene desaturase to evaluate bleaching herbicides" ZEITSCHRIFT FÜR NATURFORSCHUNG, vol. 51, no. 7-8, July 1996 (1996-07), pages 534-538, XP002112339 the whole document --- | 16 |
| X | ALBRECHT, M., ET AL.: "biochemical characterization of purified zeta-cartene desaturase from Anabaena PCC 7120 after exposure in Escherichia coli" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, 1996, pages 115-120, XP002112340 the whole document --- | 16 |
| X | BURBIDGE, A., ET AL.: "structure and expression of a cDNA encoding zeaxanthin epoxidase, isolated from a wilt-related tomato (Lycopersicon esculentum Mill.) library" JOURNAL OF EXPERIMENTAL BOTANY, vol. 48, no. 314, September 1997 (1997-09), pages 1749-1750, XP000874479 the whole document --- | 6,7 |
| Y | the whole document --- | 12 |
| | -/-- | |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/08789

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | BOUVIER, F., ET AL. : "Xanthophyll biosynthesis - cloning, expression, functional reconstruction, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (<i>Capsicum annuum</i>)" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 46, 15 November 1996 (1996-11-15), pages 28861-28867, XP002129002 the whole document --- | 6-11 |
| X | WO 96 38566 A (AGRONOMIQUE INST NAT RECH ;MARIN ELENA (FR); MARION POLL ANNIE (FR) 5 December 1996 (1996-12-05) the whole document --- | 6-11 |
| Y | the whole document --- | 12 |
| X | MARIN E ET AL: "MOLECULAR IDENTIFICATION OF ZEAXANTHIN EPOXIDASE OF NICOTIANA PLUMBAGINIFOLIA, A GENE INVOLVED IN ABSCISIC ACID BIOSYNTHESIS AND CORRESPONDING TO THE ABA LOCUS OF ARABIDOPSIS THALIANA" EMBO JOURNAL, vol. 15, no. 10, 15 May 1996 (1996-05-15), pages 2331-2342, XP002017525 ISSN: 0261-4189 cited in the application figure 4 --- | 6-11 |
| Y | figure 4 --- | 12 |
| A | BARTLEY, G.E. AND SCOLNIK, P.A.: "Plant carotenoids: pigments for photoprotection, visual attraction, and human health" THE PLANT CELL, vol. 7, July 1995 (1995-07), pages 1027-1038, XP002112110 page 1027, right column; page 1030, right column; page 1035, Table 1 --- | 1-5, 11-16 |
| P,X | FREY, A., ET AL. : "engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression" PLANT MOLECULAR BIOLOGY, vol. 39, 6 April 1999 (1999-04-06), pages 1267-1274, XP002129003 the whole document --- | 6-9,11 |
| P,X | MBEGUIE-A-MBEGUIE, D. AND FILS-LYCAON, B.: "molecular cloning and nucleotide sequence of a zeaxanthin epoxidase from apricot fruit" EMBL SEQUENCE DATA LIBRARY, 29 June 1998 (1998-06-29), XP002129004 heidelberg, germany accession no. AF071888 ----- | 6,7 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/08789

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheets

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5,11-16 partially

Isolation of gene sequences representing a maize-specific cDNA encoding Phytoene Synthase; namely SEQIDs 1, furthermore the corresponding deduced amino acid sequences SEQIDs 2.

2. Claims: 1-5,11-16 partially

Isolation of gene sequences representing a maize-specific cDNA encoding Phytoene Synthase; namely SEQIDs 3, furthermore the corresponding deduced amino acid sequences SEQIDs 4.

3. Claims: 1-5,11-16 partially

Isolation of gene sequences representing a rice-specific cDNA encoding Phytoene Synthase; namely SEQIDs 5, furthermore the corresponding deduced amino acid sequences SEQIDs 6.

4. Claims: 1-5,11-16 partially

Isolation of gene sequences representing a rice-specific cDNA encoding Phytoene Synthase; namely SEQIDs 7, furthermore the corresponding deduced amino acid sequences SEQIDs 8.

5. Claims: 1-5,11-16 partially

Isolation of gene sequences representing a rice-specific cDNA encoding Phytoene Synthase; namely SEQIDs 9, furthermore the corresponding deduced amino acid sequences SEQIDs 10.

6. Claims: 1-5,11-16 partially

Isolation of gene sequences representing soybean-specific cDNAs encoding Phytoene Synthase; namely SEQIDs 11 and 13, furthermore the corresponding deduced amino acid sequences SEQIDs 12 and 14.

7. Claims: 1-5,11-16 partially

Isolation of gene sequences representing a wheat-specific cDNA encoding Phytoene Synthase; namely SEQIDs 15,

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

furthermore the corresponding deduced amino acid sequences SEQIDs 16.

8. Claims: 6-10,11-16 partially

Isolation of gene sequences representing maize-specific cDNAs encoding Zeaxanthin Epoxidase; namely SEQIDs 17 and 19, furthermore the corresponding deduced amino acid sequences SEQIDs 18 and 20.

9. Claims: 6-10,11-16 partially

Isolation of gene sequences representing soybean-specific cDNAs encoding Zeaxanthin Epoxidase; namely SEQIDs 21,23 and 25, furthermore the corresponding deduced amino acid sequences SEQIDs 22,24 and 26.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/08789

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